CYTOLOGICAL STUDIES ON MUCUS FORMATION AND SECRETION IN BUSYCON [1]

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The formation and secretion of mucus, sometimes of great importance to physiologists and ecologists, are poorly understood. A few studies have been made of mucous glands and of their activity in various organisms. Thus, Re (1951) has studied secretion of mucus in the amphibian oviduct, and Patten (1950) has described some aspects of histogenesis in mucous epithelia of a urodele and two rodents. Vaubel (1933) observed the behavior of mammalian synovial cells in tissue culture. Studies on invertebrates have included observations on some cytological aspects of the secretion *in vivo* by an oligochaete (Millott, 1948). The mucus-producing ability of mollusks is well known; the pallial mucous cells of a pelecypod, *Anodonta*, have recently been studied by Defretin and Riff (1948). The prosobranch (gastropod) hypobranchial gland contains masses of large mucus-producing cells, and for this reason deserves special attention (Ronkin and Ronkin, 1951). Thus Tarao (1935) described the hypobranchial gland of the abalone, *Haliotis japonica*, and attempted to analyze the time-sequence of the secretion using Hirsch's (1931) methods for the classification and analysis of secretory activity. An earlier morphological description of the gastropod hypobranchial gland (Dakin, 1912) includes some reference to previous work. The present study is largely cytochemical in nature and is directed mainly toward an understanding of the chemical processes leading to formation of mucus in the hypobranchial gland of *Busycon*. Since this gland has not been adequately described, it is necessary to include a brief morphological survey; this adds to our meager knowledge of molluscan histology.

Materials and Methods

Obtaining the excised gland. *Busycon canaliculatum* (Linnaeus), a large marine snail, is easy to handle and was readily available during most of the year by dredging near Woods Hole, Mass. To obtain the fresh gland the animal was held on a board, with the columella vertical and apex pointed downward. The outer whorls of shell were cracked with a hammer; then a *dulled* axe blade was inserted into the external groove separating adjacent whorls, and twisted. After one or two whorls of shell were removed in this manner the animal was grasped at the operculum, its columellar muscle worked loose with the fingers, and the intact snail then "unscrewed" from the remaining shell. One could now make out the pallial organs

[1] This work was supported by the Faculty Research Committee of the University of Delaware and by a contract between the Office of Naval Research, Department of the Navy, and the University of Delaware (NR 160–015).

[2] With the technical assistance of Elizabeth P. Ronkin.

(rectum, hypobranchial gland, ctenidium and osphradium, from the midline to the animal's left), through the translucent roof of the pallial cavity. The portion of the mantle bearing the hypobranchial gland was excised for study.

Preparation of material for microscopic study. The excised gland was easily maintained alive in sea water at room temperature (20 to 25° C.) for five days, during which time ciliary activity and secretion of mucus were evident. Attempts to obtain separate, living mucous cells and to observe them with the compound microscope failed, probably because of the fragility of these cells. For the same reason, attempts to obtain fresh-frozen sections with the freezing microtome also failed. However, it was possible to obtain frozen sections (at 25 μ) of glandular epithelium which had previously been fixed in 10% neutral formalin for 8 to 24 hours. Some attempts to stain the living cells with dilute solutions of neutral red, Janus green B, and toluidine blue O were also made, but the dye failed in each case to penetrate the mucous layer surrounding the cells. For most histological and cytological studies the gland was placed in a fixative solution immediately after excision.

Gilson's mercuric-nitric solution gave good fixation most often when compared with other fixatives (acetone at 5° C., alcohol-formalin, Formol-Zenker, *i*-pentane at − 170° C., lead acetate-formalin, 5% mercuric chloride, osmic-sublimate,[3] propylene glycol at − 20° C., Regaud's, Susa's, and toluene at − 20° C.). Ethanol was used for dehydration, followed by clearing in toluene and infiltration and imbedding in paraffin. Sections were cut at 7 to 10 μ.

For general studies of histological and cytological features, sections were stained with toluidine blue O (0.1% aqueous) followed by potassium ferrocyanide (1% aqueous), Weigert's hematoxylin, and metanil yellow (0.25% in 0.25% acetic acid), with the usual intermediate rinses and subsequent dehydration, clearing, and mounting. Mayer's mucicarmine was occasionally used as a substitute for the toluidine blue O-potassium ferrocyanide combination, and was satisfactory for many purposes. Mallory's triple stain was used for histological features.

In addition to these, and to certain special methods described in the literature, the following methods are referred to in this paper:

a. Alcian blue 8GS.[4] This stain, whose use for mucus has recently been suggested by Steedman (1950), was employed in 1% aqueous solution; metanil yellow was used as a counterstain.

b. Amylase digestion. Hydrated sections were incubated in human saliva at 37.5° C. for one hour, then for an additional half-hour in distilled water to remove the salivary mucus.

c. Bauer-Feulgen stain. This procedure for demonstrating glycogen and mucus follows the schedule outlined by Glick (1949), using 4% chromic acid.

d. Hyaluronidase digestion. Hydrated sections were incubated in 0.01% bovine testicular hyaluronidase[5] at 37.5° C. for the desired time. They were then rinsed several times in distilled water before further treatment.

[3] Ludford-Mann-Kopsch method (Bensley and Bensley, 1938).

[4] A new dye derived from monastral fast blue B (Haddock, 1948). Samples were kindly furnished by Dr. H. A. Lubs of E. I. du Pont de Nemours and Co., Inc., and by Dr. H. F. Steedman of the University of Glasgow. It has recently become commercially available as a biological stain in the United States.

[5] Worthington Biochemical Sales Co., Freehold, N. J.

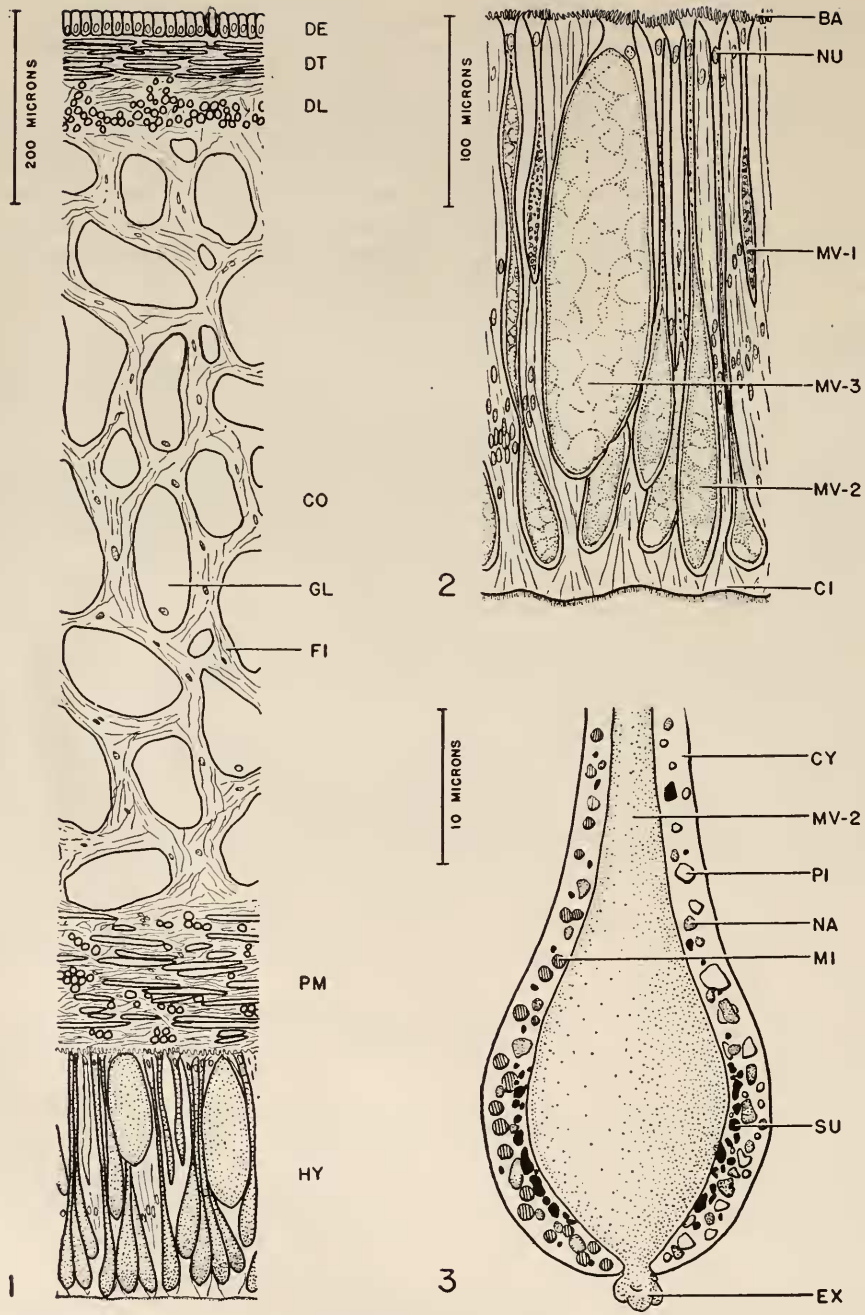

FIGURE 1. Semi-diagrammatic transverse section through a portion of the mantle of *Busycon* bearing the hypobranchial gland. CO, connective-tissue layer; DE, dorsal epithelium; DL, dorsal longitudinal muscular layer; DT, dorsal transverse muscular layer; FI, fibrous tissue; GL, glycogen cell; HY, hypobranchial gland; PM, pallial muscular layer.

e. Nadi test. This procedure for demonstrating cytochrome oxidase, using *a*-naphthol and *p*-amino dimethylaniline, is described by Glick (1949). Tissues were fixed in 10% formalin and sectioned with the freezing microtome, stained, and mounted in Apáthy's gum-syrup (Lillie, 1948).

f. Prussian-blue test for intracellular iron. Hydrated sections were immersed first in ammonium sulfide, then in potassium ferrocyanide solution (0.75% in 0.25% HCl), and finally counterstained in eosin Y, with intermediate rinses in distilled water.

When available, Commission-certified stains were used. Unless otherwise stated, stained sections were dehydrated in a series of ethanols, cleared in xylene and mounted in Clarite. When enzymatic digestion was used, comparisons were made with undigested sections from the same block.

Observations

General structure of the hypobranchial gland (Fig. 1). The glandular and ciliated epithelium which faces the pallial cavity consists of a single layer of long, slender cells, arranged normal to the free epithelial surface, and is bounded dorsally by a basement membrane. Above the basement membrane is a relatively thin sheet of fibrous and muscular tissue, with fibers arranged parallel to the free surface. The thickest layer of the mantle comes next and consists of a loose network of fibrous connective tissue. Most of the spaces bounded by the fibers are filled by cells which contain glycogen in large amounts. Next dorsally is a muscular and connective-tissue layer. Finally there is a very thin layer of glandular epithelial cells which bounds the dorsal (outer) surface of the mantle.

Examination of the inner epithelial layer (*i.e.*, the hypobranchial gland itself; Fig. 2) revealed that it is composed of ciliated cells, several types of mucous cells, and some rather interesting cellular fragments. The ciliated cells are conical. The bases of the cones pave the surface of the gland and their apices are prolonged as slender stalks whose extremities probably attach to the basement membrane. The nucleus of each ciliated cell is to be found about half the distance from the surface to the basement membrane.

Types of mucous cells. The following working classification of mucous cells is proposed:

Mucous cells of type 1 frequently extend only part way from the basement layer to the free surface of the gland. The mucous vacuole is slender and usually spindle- or club-shaped; its contents appear gray and foamy in unstained material, dark red with Mayer's mucicarmine, and frequently blue (*i.e.*, without metachromasy) with toluidine blue O.

Figure 2. Cell types in the hypobranchial gland. BA, basement membrane; CI, ciliated cells; MV–1, 2, 3, mucous vacuoles of cells of types 1, 2, and 3, respectively; NU, nucleus of a type-2 cell. Note the masses of nuclei about two-thirds the distance from the basement membrane to the ciliated surface.

Figure 3. Semi-diagrammatic drawing of the distal end of a type-2 cell. CY, cytoplasm; EX, extruded mucus; MI, mitochondrion (mitochondria are cross-hatched and are omitted from the right half of the drawing); MV–2, mucous vacuole; NA, nadi-positive granule (stippled); PI, pigment granule (omitted from the left half of the drawing); SU, sudanophilic granule (solid black). Golgi material is not represented.

Mucous cells of type 2 generally extend the full depth, or nearly the full depth, of the glandular layer. The large mucous vacuole is club-shaped, and usually can be followed from its distal enlargement almost to the base of the cell; although one or two broad regions may be seen, usually the vacuole tapers rapidly and terminates in isolated granules close to the nucleus. In some cells the nucleus is at some distance from the basement membrane; in such cells all the mucus is distal to the nucleus, and the cell is connected to the basement layer by a slender stalk. The mucus stains metachromatically (reddish violet) with toluidine blue O and bright red with mucicarmine; it appears gray and foamy in unstained material. One may suggest that cells of type 1 include the youngest in a series of mucous cells of different ages, and that type-2 cells are older; evidence in support of this relationship will be offered elsewhere in this paper.

TABLE I

Staining and other reactions of mucous-vacuolar contents in the various kinds of glandular cells described

Stain and condition	Type 1	Type 2	Type 3
a. Mayer's mucicarmine	Reddish violet	Red	Like type 2
b. Alcian blue 8GS	Blue-green, but parts of some vacuoles unstained	Blue-green	Like type 2
c. Toluidine blue O	Some blue and nongranular; some blue with fine or coarse metachromatic granules; the latter having decreasing amounts of blue-staining interstitial material; some with metachromatic, foamy contents with optically empty interstices	Reddish violet (metachromatic)	Like type 2
d. Toluidine blue O, preceded by 24 hours hyaluronidase digestion	Metachromasy in granules, but paler; blue color faded or absent	Pale metachromasy	Like type 2
e. Bauer-Feulgen	Red granules, of uniform size within each cell	Red and finely granular	Clumps of fine, pink granules
f. Bauer-Feulgen, preceded by 24 hours hyaluronidase digestion	No visible change on exposure to enzyme	Pale pink	Optically empty

Mucous cells of type 3 have very large, ellipsoidal or irregular vacuoles whose contents, when present, appear to have the same staining properties as those of type 2, but seem to be less dense or more open in texture. They are found close to the basement layer but may extend most of the distance to the surface. Whether cells of type 3 are part of the same series as those represented by types 1 and 2 cannot be stated for lack of evidence. However, the similarity between types 2 and 3 with respect to staining reactions and susceptibility to hyaluronidase (Table I) suggests chemical similarities in the mucus.

Usually the mucous cells are normal to the free surface, but some portions of the gland (especially those at its left, or ctenidial, edge) are thrown into folds which bear a superficial resemblance to the gastric crypts in vertebrates. In such locations the mucous cells usually appear to be of type 2, but they are much shorter than

elsewhere in the gland and seem to pour their secretions into the crypt rather than directly into the pallial cavity.

Masses of nuclei, paler and more elongated than those found at the bases of mucous cells, were frequently seen occupying clusters of cytoplasmic strands, at positions about halfway from the basement membrane to the free surface. Many nuclei were also encountered in the secreted mucus lying on the surface of the gland. Some of these nuclei belong to ciliated cells, but none could be traced to mucous cells. It is possible that the remaining nuclei and cytoplasmic strands are remnants of former mucous cells whose distal portions have become emptied or lost.

The contents of the mucous vacuoles. In formalin-fixed material, frozen-sectioned and unstained, the contents of the mucous vacuole are gray, refringent, and appear foamy in texture. In toluidine blue-, alcian blue-, or mucicarmine-stained cells the appearance is slightly distorted, probably owing to the preceding dehydration, and the contents tend to look fibrous. It is the fibrous phase, corresponding to the continuous or suspending phase of the "foam," which becomes stained with uniform intensity throughout the vacuole. It is thus unlikely that the suspended spheres in the mucous vacuole, which remain unstained, are of mucous nature. The mucus in the three types of mucous cells stains differently with the various staining methods used, as indicated in Table I.

Cytoplasmic inclusions other than mucus (Fig. 3). The fresh gland appears yellowish brown to the unaided eye. Microscopic examination of portions of the gland fixed in 10% formalin and sectioned with the freezing microtome revealed that the scanty cytoplasm of the stained or unstained mucous cells of all types contains many canary-yellow granules. The yellow color which they confer upon the cytoplasm is visible under low power. These granules vary widely in size, the largest being about 5 μ in diameter and the smallest at the limit of microscopic resolution in white light. The larger granules appeared to be concentrated about the distal, more bulbous portion of each type-2 cell. The following description refers to type-2 cells, which are those most commonly found.

Mitochondria were demonstrable with Regaud's hematoxylin. Spherical basophilic granules, 0.4 to 2.1 μ in diameter, were concentrated and more easily distinguishable in the distal half of the mucous cell than in the proximal, or basal, half. They were not as easily demonstrated with Altmann's aniline acid fuchsin.

The possible presence of Golgi material was shown by osmiophilia in the mucous cells of deeper portions of the "crypts" at the left edge of the hypobranchial gland. The densely packed, intensely brownish-black granules occupied the distal portion of the cytoplasm of each cell, outside the mucous vacuole. Since these sections had been bleached with potassium permanganate subsequent to osmic-acid treatment, the distribution of osmiophilic granules may indicate the location of Golgi material (Lillie, 1948).

Formalin-fixed, frozen-sectioned glandular material stained with Sudan black B (Wislocki and Dempsey, 1948) revealed blackish-green granules distributed throughout the cytoplasm, but concentrated especially in a broad belt encircling the distal portion, but not the tip of the mucous vacuole. Most of the sudanophilic granules were 0.4 to 1.3 μ in diameter, with a few as large as 2.5 μ. Several belts of granules measured were 4 to 12 μ wide, compared with mucous-vacuolar diameters

of about 17 μ in this portion of the cell. Sudanophilia is presumptive evidence of lipid nature.

Similarly fixed and sectioned material was treated with the "nadi" reagent for the determination of cytochrome oxidase. Treated sections showed intense, blue granules, usually varying in diameter from 1.7 μ downward, throughout the cytoplasm. Near the expanded distal portion of the cell, granules up to 3.5 μ in diameter were found.

Iron was not present in any of the mucous cells in amounts large enough to be detected by the Prussian-blue test. Prussian-blue granules were present, however, in the ciliated epithelium of the adjacent ctenidium, portions of which were sometimes included on the same slides with hypobranchial-gland material.

Attempts to demonstrate glycogen in the mucous cells with the Bauer-Feulgen test were unsuccessful. The presence of Bauer-Feulgen-positive material in the underlying connective tissue, and its absence in control sections which had been subjected to digestion with salivary amylase, suggested that glycogen was absent from the mucous cells.

Observations of secretion. A few cells were observed in which the secretion of mucus had been stopped by histological fixation. The freshly extruded mucus usually appeared as a small wisp protruding from the center of the distal tip of the cell, but occasionally as a small rounded cap or button covering the tip of the cell. There was no evidence of a large amount of mucus being extruded from a cell in a short time, suggesting that the production of mucus is a slow and continuous type of activity, probably varying in rate from time to time, and with secretion occurring only occasionally. Millott (1948) has seen sphincter-like structures surrounding the mouths of mucous cells in *Lumbricus;* no analogs of these structures could be seen in material studied here. It is possible that the ciliated cells, by the lateral exertion of pressure, may aid in mucus secretion in *Busycon* in the way Millott has suggested for the oligochaete, but no evidence for this mechanism was observed.

Discussion

If one defines a mucous cell for the purposes of this study as one whose secretion-vacuolar contents stain metachromatically with toluidine blue O, then it is probable that all kinds of secreting cells observed were mucous in nature. In *Haliotis,* however, Tarao (1935) found several kinds of non-mucous, secreting cells which he was able to classify according to the types of secretion granules. The non-mucous cells were intermixed with mucous, ciliated, and sustentacular cells in the hypobranchial gland. In *Busycon* all the observed glandular cells appear to be associated with mucus production, and no sustentacular cells could be distinguished, though these may have been represented by some of the massed nuclei referred to above.

The probable involvement of the nucleus in mucus formation, suggested by Tarao, is supported by the location of the nucleus near the slender base of the mucous vacuole in *Busycon.* Painter (1945) has suggested that formation of secretory materials is related to nuclear metabolism in Drosophila and in the honeybee. Wilson (1928) has referred to the earlier literature on nuclear function in relation to secretory activity in protozoa.

The genetic relationships of the different kinds of glandular cells in *Busycon* do not seem as clear as those described in *Haliotis* by Tarao. The observations re-

ported here suggest that mucous cells of types 1 and 2 are earlier and later stages in the maturation of cells of a single type. The position of cells of type 3 is uncertain; they may constitute a separate category, or they may represent an older stage than type 2, in the same series. The evidence for the relation between cells of types 1 and 2 lies in (1) the generally greater length of type-2 cells, compared with type 1; (2) the failure to observe extrusion of secretion from type-1 cells; (3) the heterogeneity of vacuolar contents of type 1, with respect to its staining reactions with alcian blue and toluidine blue, suggesting a transitional state.

The last observation confirms those of Vaubel (1933), who found that young synovial cells in tissue culture contained granules orthochromatic to toluidine blue, whereas older cells contained metachromatic granules. In *Busycon* the acquisition of metachromasy by the mucous-vacuolar contents, which is apparently in progress in type-1 cells, reflects a chemical change in the mucous precursor. The property of metachromasy is correlated in general with the binding of sulfate in polysaccharides (Lison, 1933; see also Michaelis and Granick, 1945, and Masamune *et al.*, 1947), and it seems likely that sulfate binding may be an important step in the formation of mucus in progress in cells of type 1.

The reported presence of glycogen in the mucous cells of *Anodonta* (Defretin and Riff, 1948) indicates a difference between *Busycon* and this pelecypod material. The failure to detect glycogen in the hypobranchial mucous cells and its presence in the overlying tissues suggest that the mucous cells may obtain their carbohydrates by diffusion from the neighboring cells. The presence of galactose in other molluscan tissues (Bell and Baldwin, 1941; Masamune *et al.*, 1947) may mean that galactogen is of general importance in this group of animals, in addition to glycogen; galactokinase and galactose-isomerase systems might well be sought.

Finally, the peculiar distribution of lipid material in relation to the mucous vacuole in type-2 cells suggests that lipid metabolism may be involved in the formation or secretion of mucus, or both. Although the granules which appeared in these cytochemical tests may well be crystalline precipitates and may not exist in the living cell, their locations suggest the distribution of constituents present in life. The distribution of mitochondria and that suggested for cytochrome oxidase may indicate the loci of biochemical systems involving aerobic syntheses. Studies on lipid transformations and respiratory metabolism in these cells and further studies of the spatial distribution of the participating substances may clarify the mechanisms of the formation and secretion of mucus.

Summary

1. The hypobranchial (mucous) gland of *Busycon canaliculatum* (L.) is located in the pallial cavity. It contains ciliated cells and three types of mucous cells.

2. Mucous cells of type 1 are thought to be precursors of type 2 because of the relative sizes and positions of the two types of cells, and because mucous vacuoles of type-1 cells appear to have varying degrees of resemblance to those of type-2 cells. Evidence concerning the relationships of type-3 cells is lacking.

3. The commonest type of mucous cell (type 2) is long and slender with a bulbous tip, one or two swellings along its length, and a basal nucleus. Most of the cell is occupied by a mucous vacuole, whose contents stain metachromatically (reddish violet) with toluidine blue O, red with mucicarmine, blue-green with alcian blue 8GS, and are partly digested by hyaluronidase. The surrounding cytoplasm con-

tains yellow pigment granules, mitochondria, Golgi apparatus, and nadi-positive granules. The distribution of sudanophilic material is peculiar in that a belt of lipid granules surrounds the sub-distal portion of the bulbous tip of the vacuole. No glycogen could be demonstrated in these cells.

4. It is possible that the activities of mucous cells require diffusion of carbohydrate materials from adjacent cells. The results of the cytochemical tests suggest that aerobic utilization of carbohydrates (possibly galactose) or lipid materials, and the binding of sulfate groups may be of importance in the formation of mucus from its precursors.

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